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Cyclopia subternata growth, yield, proline and relative water content in response to water deficit stress

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Cyclopia, generally known as honeybush, and belonging to the *Fabaceae* family, originates from the Cape Floristic Region of the Eastern Cape and Western Cape provinces of South Africa. Currently, 6 honeybush species are commercially cultivated but, to date, there have been limited trials attempting to study their agronomic water demand. A pot trial was conducted where *Cyclopia subternata* plants were cultivated on different soil types (Stellenbosch granite, Stellenbosch shale and Stellenbosch clovelly) and subjected to three different water-deficit stress levels (well-watered, semi-stressed and stressed). Remarkably, irrigation treatments and soil types did not significantly affect the growth of the plants. However, the well-watered treatment consistently had higher yields compared to the other two treatments. The water-stressed (semi-stressed and stressed) treatments had lower relative water contents (RWC) with higher concentrations of proline, which signify water stress, compared to the control treatment. Higher proline and lower RWC contents found in this study are indications of water stress.

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INTRODUCTION

South Africa, a drought-prone country, is home to rooibos (*Aspalathus linearis*), bush (*Athrixia phylicoides*) and honeybush (*Cyclopia* species) teas (Joubert et al., 2011). The teas are sold as either black or green (fermented or unfermented, respectively) (Horn, 2019). Even though the commercialization of some of these remedial teas is still in its infancy stage, honeybush has gained recognition, while rooibos is the most well-known and well established in the industry (Van Wyk and Gericke, 2000; Joubert et al., 2008; Joubert et al., 2011).

Studies state that these South African indigenous tea species have essential nutrients (iron, calcium, magnesium, copper, and potassium) that can improve wellbeing and/or prevent diseases, and have economic potential (Rampedi and Olivier 2005; McGaw et al., 2007). These herbal teas are famous for their rich caffeine-free and organic antioxidant properties, which are helpful in colon, throat and lung illnesses, prevention of urinary stone and tooth caries and other medical problems (Soni et al., 2015).

The demand for honeybush tea has prompted concerns of over-exploitation of natural populations of the *Cyclopia* species. The increased rate of wild harvesting diminishes the natural population, thus making the exploitation of *Cyclopia* species unsustainable. Harvesting practices have contributed to the decrease and even disappearance of populations of the wild *Cyclopia* species (Du Toit et al., 1998). Other factors threatening the growth of the honeybush industry include drought and veld fires. To ensure sustainable production, commercial honeybush plantations have been established (Joubert et al., 2011).

Commercial production is therefore becoming increasingly important to save the natural populations from decline while ensuring consistent supply. Cultivation of *Cyclopia* species will not only contribute to sustainability and conservation of the species but will also improve the livelihoods of rural harvester communities. Although cultivated honeybush plants receive water through irrigation in addition to rainfall, irrigation volume is at the discretion of farmers, without any understanding of the water requirements of the species. Presently, the shortage of water has massively increased in some parts of the world, including some regions in South Africa, due to a variety of reasons such as an ever-increasing population, industrialization, water pollution and poor management, climate change and others (WWAP, 2012; Connor, 2015; Long and Pijanowski, 2017).

In addition, the South African Department of Water and Sanitation (DWS) has reduced agricultural allocations significantly, and irrigation volume for the agricultural sector is unlikely to increase anytime soon. For example, in 2015, DWS restricted an irrigation water allocation in KwaZulu-Natal by 40–100% due to a water shortage caused by insufficient rain (RSA, 2015). Also, agriculture in the Western Cape has had to cut its water use by 60% since 2017 (WWF, 2018). As a result, research that focuses on the sustainability of water-use in agriculture is gaining huge interest (Velasco-Muñoz et al., 2018). Environmental factors, including water stress, tend to interfere with crucial physiological processes and biochemical mechanisms; resulting in yield loss (Per et al., 2017).

Therefore, research on water-use and the effects of stress on plant growth is crucial for production sustainability in agriculture (Harb et al., 2010). Plants have proven to use protective mechanisms such as proline and carbohydrate accumulation to cope with water-deficit situations (Mabizela, 2020). Proline is a water-soluble amino acid and beneficial solute that accumulates in plants under different kinds of stresses, such as drought, cold, heat, heavy metal, nutrient, and salt stress (Siddique and Dubey, 2017).

Relative water content (RWC) is a useful measure of plant water status in terms of the physiological consequences of cellular water deficit and may indicate the degree of water stress expressed under drought and heat stress (Surendar et al., 2013; Soltys-Kalina et al., 2016). It combines leaf water potential and the effect of osmotic regulation to quantify plant water status (Lugojan and Ciulcas, 2011; Kardile et al., 2018). Insufficient water in plants due to stress results in low RWC (Chakhchar et al., 2015).

A plant's ability to retain turgor during water-deficit periods guarantees smooth metabolic processes for growth (Čereković et al., 2013). Several studies have stated that RWC determination is an efficient method of assessing drought tolerance and plant water status (Slabbert and Krüger, 2004; Li-Ping et al., 2006; Jones, 2007; Obidiegwu et al., 2015). To date, limited studies have been conducted to investigate the water needs of *Cyclopia* species. Therefore, the aim of this study was to evaluate the effects of 3 different irrigation treatments on growth, yield, proline and relative water content of *Cyclopia subternata* species of honeybush.

METHODS AND MATERIALS

Experimental site and layout

A greenhouse pot trial was conducted at the Agricultural Research Council (ARC), Infruitec-Nietvoorbij (latitude −33.914395° and longitude 18.861390°) in Stellenbosch, South Africa, to determine the effect of water stress on *C. subternata*. The experiment was conducted for 140 days (from end-July to mid-December 2020). The experimental design was a randomised block design (RBD) with 9 treatment combinations (irrigation x soil type) replicated at random in each of 4 block replicates. The treatment structure was a 3 x 3 factorial with 3 irrigation levels (well-watered, semi-stressed and stressed) and 3 soil types (Stellenbosch granite, Stellenbosch shale and Stellenbosch clovelly).

Soil collection, preparation, and planting

Soil collection was carried out from three different sites at the ARC Nietvoorbij research farm. For each site, soil samples were collected from the 0–30 cm soil depth, sieved with a 3 mm sieve to remove large fragments, followed by baseline physicochemical analysis of the composited samples at a commercial laboratory (Bemlab, Strand). 14 kg of soil was weighed into a 30 cm plastic pot, using a digital scale. The soil in each pot was irrigated to pot capacity (PC) before planting. Nine-month-old honeybush (*C. subternata*) seedlings were transplanted to one plant per pot. The plants were well-watered for 5 weeks to ensure good establishment before introducing the different irrigation treatments.

Irrigation and weed control

From the 6th week after transplanting (WAT), *C. subternata* plants were subjected to 3 different irrigation treatments for 105 days (September–December 2020). The well-watered treatment (control) received 500 mL of water 3 times a week, semi-stressed received the same quantity of water twice a week while the stressed treatment received 500 mL of water once a week until the end of the study. The plants were hand irrigated with an Erlenmeyer flask. Weeds that emerged in the pots during the trial period were mostly broad-leaved plants. The weeds were either hand-pulled or manually removed using a garden fork immediately after irrigation when the soil was still wet.

Data collection

Growth parameters

Measurement of growth parameters commenced at 6 WAT on a monthly basis, until the trial was terminated in December 2020. Plant height was measured from the soil surface to the tip of the longest shoot, using a tape measure, stem diameter was measured with a digital Vernier caliper while the stem circumference was calculated from stem diameter values using the following formula:

$$C = \pi d \quad (1)$$

where π = 3.14 and d = diameter

Total yield (shoot and root biomass)

At the end of the study (20 WAT), the above-ground biomass (shoot) was cut just above the soil surface using a pruning shear, placed in a labelled paper bag and then weighed using a sensitive weighing balance to obtain the fresh mass of the shoot. The fresh shoot was oven-dried at 70°C for 24 h. The dried samples were also weighed and recorded using a sensitive digital scale to 4 decimal places. The root biomass was determined by washing plant roots from each pot under running water using a 0.053 mm sieve in order to separate the roots from the soil and prevent loss of fine roots. The washed roots were air-dried overnight and weighed using a sensitive scale. Total plant yield is the combined fresh weight of the above-ground biomass and the air-dried root biomass.

Estimation of proline content using the colorimetric method

Determination of the proline content of *C. subternata* commenced at 6 WAT using the modified method of Ábrahám et al. (2010). Leaf samples were collected at 2-week intervals during the growth period. The extraction procedure was done by crushing 0.1 g of fresh frozen leaves in 0.5 mL of 3% sulfosalicylic acid (w/v), using a plastic test tube and pestle. The homogenised extracts were centrifuged for 5 min at a speed of 13 500 r/min. A reaction mixture of 0.1 mL of 3% sulphosalicylic acid, 0.2 mL of glacial acetic acid, 0.2 mL of acid ninhydrin buffer (1.25 g ninhydrin, 30 mL glacial acetic acid, and 20 mL of 6 M phosphoric acid) and 0.1 mL of centrifuged sample extract was made in a test tube with a pipette. The mixture was boiled for 30 min at 100°C then terminated in an ice bath at room temperature. After complete cooling, 1 mL of toluene was added to the mixture and mixed thoroughly, then placed on the bench for 5 min to allow separation of chromophore. The absorbance was read at 520 nm on the UV-visible spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, Waltham MA, USA) with a 10 mm quartz glass cuvette. From the proline standard curve, the proline concentrations of the *C. subternata* samples were determined. Proline content was calculated using the formula:

$$\text{Proline content (mmoles/g)} = \frac{\text{proline (mg/mL)} \times \text{toluene (mL)}}{115.5} \times \frac{5}{\text{sample mass (g)}} \quad (2)$$

where 115.5 = molecular mass of proline

Determination of relative water content (RWC)

An improved version of the method of Sade et al. (2015) was used to determine the RWC of *C. subternata* leaves. Leaf samples were collected fortnightly at midday (12:00) where 5 top-most leaves per plant were collected, cut into two halves and immediately stored in pre-weighed, labelled glass vials to minimize humidity or vapour loss. The samples were preserved on ice during sampling and quickly transported to the laboratory for RWC determination. Fresh weight (FW) of each sample was determined using a sensitive weighing scale. 2 mL of distilled water was added to each vial, kept in a dark cupboard at room temperature for 4 h to facilitate re-hydration. Thereafter, the turgid leaf samples were removed from the vials and slightly blotted with a paper towel to remove excess water. The blotted leaves were weighed to determine the turgid weight (TW) and later oven-dried at 70°C for 48 h. The dried samples were later weighed to determine the dry weight (DW). Relative water content was calculated using the formula shown below:

$$\mathrm{RWC} = \frac{(\mathrm{FW} - \mathrm{DW})}{(\mathrm{TW} - \mathrm{DW})} \times 100 \quad (3)$$

Statistical analysis

Data were analysed with the randomised block factorial ANOVA using SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC, USA, 2013). ANOVA was used for each observation time (harvest/month) separately, as well as with time as sub-plot factor (Little, 1972). The Shapiro-Wilk test was utilized in testing for deviation from normality (Shapiro, 1965). Fisher's least significant difference was calculated at the 5% level to compare treatment means (Ott, 1998). A probability level of 5% was considered significant for all tests.

RESULTS AND DISCUSSION

Physical and chemical characteristics of the soils

The results of the baseline physicochemical analysis of the soils on which the *C. subternata* plants were grown are shown in Tables 1 and 2. Stellenbosch granite soil had the highest coarse sand levels (0.5–2 mm) while the lowest was found in Stellenbosch shale. Stellenbosch clovelly had more clay content, with Stellenbosch granite having the lowest (Table 1). The textural classes for Stellenbosch granite, Stellenbosch clovelly and Stellenbosch shale fall within the coarse sandy loam, fine sandy clay loam and sandy clay loam, respectively. Soil pH and other soil nutrients were within the range for normal growth of most plants.

Growth parameters

In general, water stress and soil type had no significant influence ($p > 0.05$) on plant height, stem diameter or stem circumference throughout the period of the trial (Fig. 1, Table 3). A summary of *p*-values for separate ANOVAs of growth parameters per month is presented in Table 4.

When compared to the stressed plants, the well-watered (control) treatment had significantly taller plants with greater stem circumference in the first sampling month on Stellenbosch clovelly soil. Thereafter, growth and development of plants did not differ significantly among treatments. The results for the growth parameters of *C. subternata* in this study contrast with the findings of Tshikhudo et al. (2019) where plant height, stem diameter and the number of leaves of bush tea (*Athrixia phylocoides* DC). increased with increase in rainfall.

Stress experienced by crops during growth has a cumulative effect, which ultimately reduces the final biomass production (Kamara et al., 2003). This may be the reason why there was generally no significant difference in the growth of *C. subternata* grown in this trial while the cumulative effects of water stress were only seen in the harvested biomass (Table 5 and Fig. 2). However, a study by Habibi (2018) on *Aloe vera* demonstrated that short-term water deficit had no significant effect on the leaf biomass. The short duration of the present study may be responsible for the non-significant differences observed in the growth of both the drought-stressed (semi-stressed and stressed) and the well-watered (control) plants.

Table 1. Baseline physical characteristics of the three types of soil used in the study

Physical characteristics	Stellenbosch granite	Stellenbosch shale	Stellenbosch clovelly
Clay (<0.002 mm)	13	20	23
Silt (0.002–0.02 mm)	17	13	6
Fine sand (0.02–0.2 mm)	33	50	37.8
Medium sand (0.2–0.5 mm)	3	5	13.0
Coarse sand (0.5–2 mm)	35	12	20.4
Stone volume (%)	0.22	7.72	0.00
Soil textural class	Coarse sandy loam	Fine sandy clay loam	Sandy clay loam

Table 2. Baseline chemical composition of the three soil types

Soil type	Ex. cations (cmol (+)/kg)				Macronutrients				pH (KCl)	Resistance (Ω)	K (%)	Ca (%)	Na (%)	Mg (%)	Acid saturation (%)
	Na	K	Ca	Mg	NO_{3-}	P	NH_4^+	K							
SG	0.14	0.52	4.4	1.6	31.3	23.9	3.2	203	5.3	800	6.97	58.99	1.88	21.45	10.71
SC	0.13	0.52	4.7	1.2	39.7	29.6	3.3	205	5.5	910	7.17	64.82	1.79	16.55	9.67
SS	0.07	0.32	2.8	0.59	10.6	16.9	13.4	124	5.5	1 400	7.59	66.38	1.66	13.99	10.39

SG = Stellenbosch granite; SC = Stellenbosch clovelly; SS = Stellenbosch shale

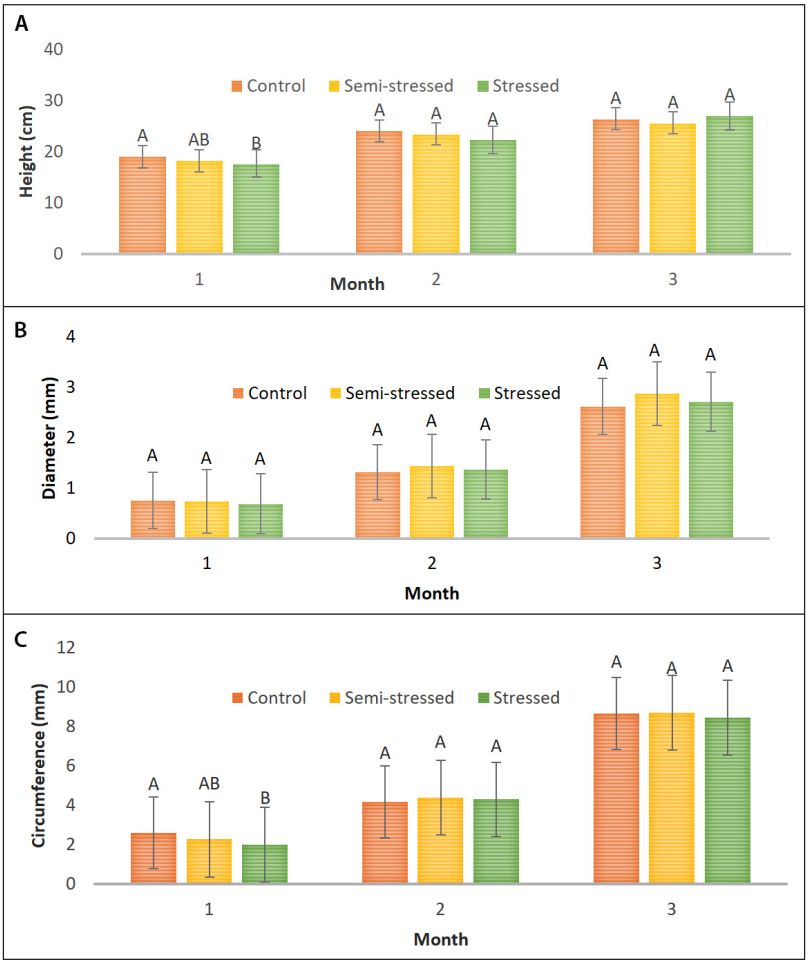

Figure 1. Effects of different irrigation levels on (A) plant height, (B) stem diameter and (C) stem circumference of *C. subternata* in different months. Means with the same letters are not significantly different ($p \leq 0.05$). Whiskers = standard error bars.

Table 3. Mean growth of *C. subternata* established on three different types of soil at different sampling times

Sampling time	Soil type	Plant height (cm)	Stem diameter (mm)	Stem circumference (mm)
1	Stellenbosch granite	17.9083 ± 1.79 [a]	0.69659 ± 0.10 [a]	2.2632 ± 0.41 [a]
	Stellenbosch shale	18.4510 ± 1.99 [a]	0.69737 ± 0.09 [a]	2.4456 ± 0.63 [a]
	Stellenbosch clovelly	18.6031 ± 1.52 [a]	0.78639 ± 0.23 [a]	2.1376 ± 0.39 [a]
	LSD	1.38	0.13	0.36
2	Stellenbosch granite	23.248 ± 2.58 [a]	1.3674 ± 0.26 [a]	4.3648 ± 0.54 [a]
	Stellenbosch shale	22.801 ±2.75 [a]	1.3098 ± 0.25 [a]	4.3931 ± 0.86 [a]
	Stellenbosch clovelly	23.729 ± 3.45 [a]	1.4395 ± 0.23 [a]	4.0709 ± 0.86 [a]
	LSD	2.51	0.22	0.61
3	Stellenbosch granite	26.557 ± 4.54 [a]	2.7348 ± 0.52 [a]	8.8084 ± 1.12 [a]
	Stellenbosch shale	25.897 ± 3.75 [a]	2.6197 ± 0.50 [a]	8.6203 ± 1.66 [a]
	Stellenbosch clovelly	26.515 ± 5.61 [a]	2.8529± 0.41 [a]	8.3426 ± 1.71 [a]
	LSD	4.25	0.43	1.22

There is no significant difference (p ≥ 0.05) among treatments per sampling time. N = *12; LSD = least significant difference. Data are mean ± standard deviation.*

Table 4. Summary of *p*-values for separate ANOVAs of growth parameters per month

Effect	Df	Plant height			Stem diameter			Stem circumference		
		1	2	3	1	2	3	1	2	3
Rep	3	0.6831	0.3165	0.5993	0.1744	0.6363	0.5498	0.1988	0.3037	0.1943
Irrigation	2	0.1358	0.3525	0.7881	0.5454	0.4991	0.4634	0.0084	0.75500	0.8855
Soil	2	0.5587	0.9875	0.9375	0.2916	0.4898	0.5370	0.2335	0.4934	0.7318
Irrigation x soil	4	0.0948	0.6838	0.6838	0.7972	0.7724	0.7989	0.7940	0.0957	0.1535

1, 2, 3 = sampling months; N = *12*

Yield

The results of the effect of irrigation treatments and soil type on the yield of *C. subternata* are presented in Fig. 2 and Table 5, respectively. A summarised presentation of *p*-values for separate ANOVAs on shoot and root biomass is shown in Table 6.

All three irrigation treatments significantly affected the yield (fresh and dry shoots) ($p \leq 0.05$). Highest shoot and root yields were recorded in the control treatment on Stellenbosch shale soil, with a progressive yield decline observed with increase in stress level. However, there were no significant differences ($p > 0.05$) in the root yield of the well-watered (5.05 g) and the semi-stressed (4.33 g) treatments. Stellenbosch clovelly consistently had poor shoot and root yields among the three soil types while there were no significant differences ($p > 0.05$) between the biomass yields from Stellenbosch granite and Stellenbosch shale.

Eziz et al. (2017) noted that plant growth and biomass production generally decrease with decrease in water availability. However, plants may behave contrary to this, where the cumulative effect of stress during growth may only be visible in the reduced biomass yield (Kamara et al., 2003); which was also observed in this study.

According to Khan et al. (2018), water stress can cause a severe reduction in crop yield, and both the severity and duration of the stress are critical. Water availability is a key factor for sustainable crop production. Its scarcity can have an adverse effect on the physiological and biochemical processes of the plants, thereby causing low yield. The drought-induced yield (root, fresh and dry shoot) decline was comparable to the findings of Zhao et al. (2006), who found that there was a severe reduction in the fresh and dry weights of *Brassica napus* under water-limiting conditions. Stress at the vegetative stage of plants may lead to reduced stomatal conductance, net photosynthesis and yield (Kerepesi and Galiba, 2000; Fathi and Tari, 2016). The observed yield reduction due to water stress in this study may, therefore, be attributed to impairment of physiological and biochemical processes like photosynthesis, respiration, translocation, ion uptake, and carbohydrate and nutrient metabolism (Ali and Anjum, 2016) during growth. During the period of stress, plants adopt coping mechanisms such as stomatal closure. However, stomatal closure prevents the intake of CO_2 into the plant cells, thereby interfering with the Calvin cycle, which will eventually reduce the potential yield of the crop (Ali and Anjum, 2016).

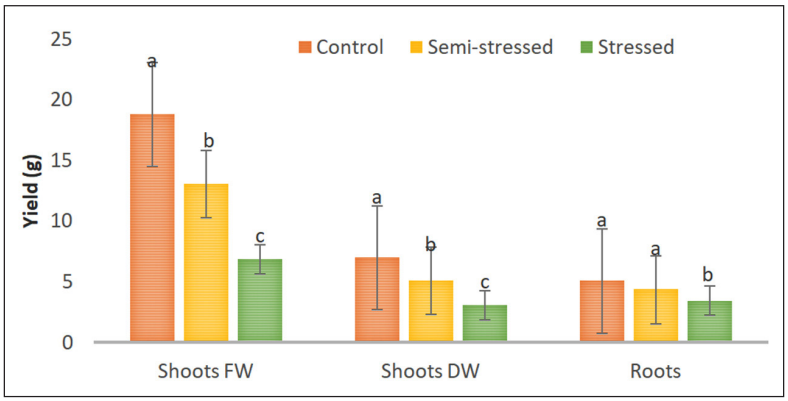

Figure 2. Effect of diverse water stress levels on the root and shoot biomass of *C. subternata*. FW = fresh weight; DW = dry weight. Means with the same letter are not significantly different ($p \leq 0.05$). Whiskers = standard error bars.

Table 5. Effect of different soil types on the yield of *C. subternata*

Soil type	Shoot weight (g)		Root weight (g)
	Fresh	Dry	
Stellenbosch granite	14.048 ± 6.20 [a]	5.0531 ± 2.12 [ab]	4.3146 ± 1.63 [ab]
Stellenbosch shale	15.625 ± 7.43 [a]	6.2229 ± 2.57 [a]	4.8604 ± 1.37 [a]
Stellenbosch clovelly	9.022 ± 5.27 [b]	3.8219 ± 2.03 [b]	3.6188 ± 1.38 [b]
LSD	3.41	1.39	0.81

FW = fresh weight; DW = dry weight. N = *12; LSD = least significant difference; means with the same letter are not significantly different* (p ≤ *0.05). Data are mean ± standard deviation.*

Table 6. Summary of *p*-values for separate ANOVAs on shoot and root biomass

Effect	df	Shoots (FW)	Shoots (DW)	Roots
Rep	3	0.7772	0.7974	0.0002
Irrigation	2	<0.0001	<0.0001	0.0013
Soil	2	0.0014	0.0061	0.0145
Irrigation x soil	4	0.3941	0.5658	0.3069

N = *12; FW = fresh weight; DW = dry weight*

Relative water content (RWC)

RWC is the proportion of water in a leaf, expressed as the percentage of its maximum volumetric water capacity at full turgor (Blum, 2011). It has a direct connection with soil water content and is mostly used as an indicant of water stress in plant leaves. Changes in leaf RWC due to the different irrigation levels in this study are depicted in Fig. 3. At both sampling times, the well-watered treatment consistently had significantly higher ($p \leq 0.05$) RWC (87% and 86%, respectively), while the stressed treatment recorded the lowest values (79% and 76% respectively), although there was no significant difference between the semi-stressed (81% and 82%) and the stressed treatments ($p > 0.05$).

Mabizela (2020) reported similar results, where the stressed and semi-stressed plants had lower RWC compared to the well-watered treatment with the variance showing from the third day after stress initiation. The low RWC in the stressed treatment indicates a stressed plant population compared with the control. Lower RWC in stressed *C. subternata* leaves used in this study is in accordance with the findings of studies on other species (Arjenaki et al., 2012; Kabbadj et al., 2017). A study on olives supports the outcomes of this research, where the lowest RWC values were reported for severely water-stressed olives (Boussadia et al., 2008). Higher RWC in plant leaves means that the plants had the least water stress, and vice versa.

At the onset of drought, a reduction in stomatal conductance can reduce availability of CO_2 for photosynthesis, subsequently leading to inhibition of underlying biochemical processes such as Rubisco carboxylation and electron transport activity, and reducing relative water content and even pigment content (Khalil et al., 2020). The reduction in the leaf RWC due to the strain caused by limited water availability may be attributed to reduction in stomatal conductance after stomatal closure in response to drought stress. As a result of this, there is an observed decrease in the RWC of the stressed *C. subternata* plants compared to the well-watered plants (Boussadia et al., 2008).

For the three soil types, in the first sampling period, there were no significant differences in the leaf RWC of *C. subternata* grown on granite and clovelly soil types ($p > 0.05$). The same observation was made for the comparison between granite- and shale-derived soils. However, water stress significantly decreased ($p \leq 0.05$) the relative water content of plants grown in Stellenbosch clovelly when compared with Stellenbosch shale (Table 7). In contrast, the second sampling time showed no significant differences among the treatments. A summary of the *p*-values for ANOVAs for relative water content per period is presented in Table 8.

Soil texture is highly influential for water uptake, and may impede root elongation, availability of water, oxygen and nutrients (Khalil et al., 2020). The high percentage of clay in clovelly soil may be responsible for the low leaf RWC in the first sampling period. High clay content in soil increases soil hardness and strength when soil is drying out. As soil strength increases, the more difficult it is for plant roots to access water and nutrients, hence, the lowest RWC in the leaves of *C. subternata* plants growing on clovelly soil. However, in the second sampling period, since this was a pot experiment, the packaging of the soil might have altered the actual field structure, allowing more macropores in the soils with high clay content than is likely to exist in the field (Khalil et al., 2020). The presence of these macropores may have contributed to the non-significant effects observed among all treatments in response to the water treatment.

Table 7. Effects of soil type on relative water content of *C. subternata* at different sampling times

Sampling time	Soil type	RWC (%)
1	Stellenbosch granite	82.766 ± 7.31^{ab}
	Stellenbosch shale	86.362 ± 7.66^{a}
	Stellenbosch clovelly	78.400 ± 9.81^{b}
	LSD	5.16
2	Stellenbosch granite	84.764 ± 8.24^{a}
	Stellenbosch shale	80.241± 7.57^{a}
	Stellenbosch clovelly	79.450 ± 9.65^{a}
	LSD	5.99

RWC = relative water content; N = *12; LSD = least significant difference. Means with the same letter are not significantly different* (p ≤ *0.05). Data are mean ± standard deviation.*

Table 8. Summary of *p*-values for ANOVA of relative water content per month

Effect	Df	RWC (%)	
		1	2
Rep	3	0.0445	0.4147
Irrigation	2	0.0072	0.0062
Soil	2	0.0145	0.1639
Irrigation x Soil	4	0.0146	0.1311

1, 2 = sampling time; N = *12; RWC = relative water content*

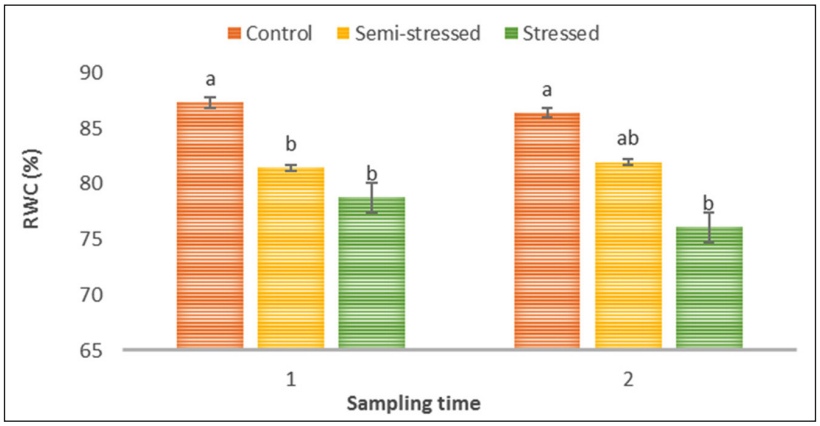

Figure 3. Relative water content of *C. subternata* in response to three different irrigation treatments at different sampling times. RWC = relative water content. Means with the same letter are not significantly different ($p \leq 0.05$). Whiskers = standard error bars.

Proline

Several abiotic factors, such as water stress, high temperatures and salinity, can cause protein modification, membrane injury and osmotic stress in plants (Meena et al., 2019). Plants respond to water stress by building-up osmolytes such as proline, glycine betaine, glycerol and many more, in order to minimize and tolerate cell injury (Sharma et al., 2019). Figure 4 shows that the stressed *C. subternata* plants in this study consistently had significantly higher proline contents in all sampling periods, while the lowest proline content was found in the well-watered ($p \leq 0.05$) plants. Significantly higher proline content was observed in the stressed plants compared to the other two treatments in the first sampling period. However, no significant difference was observed among all treatments in the second and third sampling periods ($p > 0.05$).

The obtained results are comparable to those reported by Mabizela (2020) on proline contents of *C. subternata*, where proline concentration increased massively in stressed treatments, increased slightly in semi-stressed plants, and was constant in control treatments. Higher proline content were also reported in wheat, *Amaranthus* species and *Achillea* species after being subjected to water stress (Keyvan, 2010; Slabbert and Krüger, 2014; Gharibi et al., 2016). Low proline content in plants indicates minimum water stress, and vice-versa. The high and significant levels of proline that were observed between treatments during the third sampling period may be attributed to the plants having reached the reproductive stage and having started flowering. Proline can accumulate in plants under both stress and non-stress conditions, although it is produced at low levels in all tissues in unstressed conditions (Kavi Kishor et al., 2015). As a metabolite and signal molecule, proline plays a crucial role in the synthesis of protein and the response of plant cells to environmental stresses (Mattioli et al., 2009). Proline levels may increase during wounding and pathogen attack in some tissues, different stages of plant growth and development, nodule formation, fertilization, cytokinesis, apoptosis, senescence, and cell wall lignification. Under normal physiological (un-stressed) conditions, plants accumulate high amounts of proline during the transition to flower initiation (Kavi Kishor et al., 2015), thus suggesting that proline may have a role to play in flower initiation and its subsequent development. Soil type did not have any significant effect ($p > 0.05$) on the proline contents of the plants (Table 9). A summary of *p*-values for ANOVAs of the accumulation of proline per month is presented in Table 10.

Table 9. Proline content of *C. subternata* cultivated on three different types of soil at different sampling times

Sampling time	Soil type	Proline (µmol/g FW)
1	Stellenbosch granite	25.818 ± 26.42
	Stellenbosch shale	21.996 ± 19.42
	Stellenbosch clovelly	33.053 ± 26.18
	LSD	17.95
2	Stellenbosch granite	24.258 ± 9.89
	Stellenbosch shale	22.562 ± 16.08
	Stellenbosch clovelly	30.284 ± 19.57
	LSD	11.61
3	Stellenbosch granite	37.616 ± 21.31
	Stellenbosch shale	31.220 ± 16.90
	Stellenbosch clovelly	39.156 ± 26.20
	LSD	18.77

There is no significant difference (p ≥ 0.05) among treatments per sampling time. N = *12; LSD = least significant difference. Data are mean ± standard deviation.*

CONCLUSION

From this study, it is evident that different deficit irrigation levels and soil type had no significant effects on growth parameters of *C. subternata*. Likewise, soil type had no impact on the proline, RWC and the yield of the plants. Water stress increased the proline content, resulting in lower RWC. However, deficit irrigation had a significant effect on the yield (root, fresh and dry shoot biomass). The higher the water stress, the lower the shoot and root biomass yield and vice-versa. Although, the well-watered and the semi-stressed plants gave higher shoot yield, more research is still needed to determine the tea quality of the stressed and unstressed *C. subternata* plants.

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Table 10. Summary of *p*-values for ANOVA of the accumulation of proline per period

Effect	df	Proline (µmol/g FW)		
		1	2	3
Rep	3	0.0724	0.135	0.2323
Irrigation	2	0.0595	0.0287	0.5083
Soil	2	0.4465	0.3688	0.6566
Irrigation x soil	4	0.3799	0.3793	0.8046

1, 2, 3 = sampling months; N = *12*

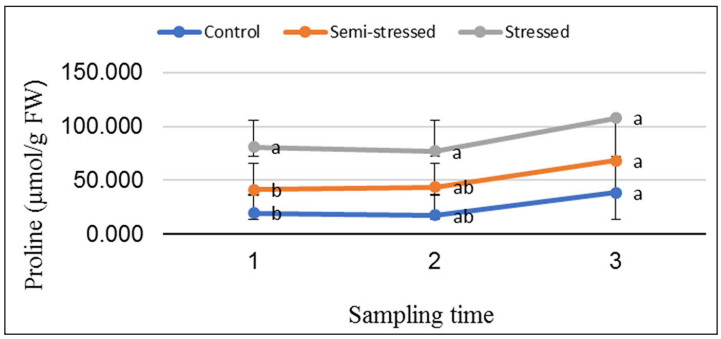

Figure 4. Effects of three irrigation levels on proline content of *C. subternata* at different sampling times. Means with the same letter are not significantly different ($p \leq 0.05$). Whiskers = standard deviation bars.

CONTRIBUTION OF AUTHORS

Mary-Jane Seji Mahlare – data collection, sample analysis, writing the initial draft, writing revision; Dr Muinat N Lewu – conceptualization, methodology, validation, student supervision, writing revision, project leadership, project management; Prof Francis B Lewu – conceptualization, methodology, validation, writing revision, student supervision; Dr Cecilia Bester – conceptualization, methodology, project leadership, project management, funding acquisition.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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